Variation in Expression of Na+ K+ ATPase
Alpha and Beta Subunit mRNAs in
Rat Tissues and Nervous System Cell Lines

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YALE UNIVERSITY

1989

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Susan Lynn Garetz

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ABSTRACT

Variation in Expression of Na^+K^+ATPase α and β Subunit mRNAs

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The expression of the Na^+K^+ATPase α and β subunits has been examined in
rat tissues at several stages of development and in a number of cell
lines derived from components of the rat central nervous system.
Significant differences in expression were seen in the tissue and
developmental specificity of the genes encoding three α subunit
isoforms. The transcript encoding the α1 isoform was seen in all of the
tissue types examined whereas the transcripts encoding the α2 and α3
isoform mRNAs were expressed predominantly in brain. A varied pattern
of mRNA expression was also seen for the β subunit which did not
correspond to the patterns seen with any of the α subunits. Transcripts
of each of the three α subunit isoforms were also expressed in all of
the cell lines derived from the nervous system. The β subunit was
expressed, however, only in a rat pheochromocytoma cell line. These
patterns of expression suggest that the various isoforms of the
Na^+K^+ATPase may perform specialized functions in diverse tissues and
during various stages of development, and that their regulation is
controlled in part by factors other than the level of mRNA expression.

ACKNOWLEDGEMENTS

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1. INTRODUCTION

The Na+, K+-activated adenosine triphosphate phosphatase
($Na^+K^+ATPase$) is an integral membrane protein found in most animal cells
that uses energy derived from ATP hydrolysis to transport Na^+ and
K^+ across the cell membrane against their concentration gradients. The
enzyme is composed of two polypeptides. The α subunit is a polypeptide
of $M_r \approx 100,000$ and contains the site of ATP hydrolysis.[1] It is thus
responsible for the enzyme's catalytic activity. In addition, the α
subunit contains the binding site for the cardiac glycosides.[1] The
smaller β subunit is a glycosylated protein of $M_r \approx 55,000$ whose function
is currently unknown.[1] It is commonly assumed that the active form of
the enzyme in cell membranes is some multiple of an $\alpha_2\beta_2$ tetramer but
the exact subunit stoichiometry remains in dispute.

For a number of years investigators have published data consistent
with the existence of more than one form of the $Na^+K^+ATPase$ enzyme. It
has been shown that astrocytes possess a low affinity K^+ uptake not seen
in neurons.[2] These glial cells also display marked osmotic swelling
when treated with ouabain.[2] Tobin showed that the dissociation of
ouabain from $Na^+K^+ATPase$ differed within a given species and that in
guinea pig brain the plotted dissociation curve was nonlinear,
suggesting that forms of the enzyme with different functional properties
could exist in the same animal.[3] Erdmann and Schoner also reported
different affinities for ouabain by the $Na^+K^+ATPase$ enzyme.[4] The
advent of techniques that allowed the preparation of relatively pure

fractions of neurons and glia enabled Henn et al. to demonstrate that glial cell ATPase was always between two and four times more active than the neuronal enzyme as well as markedly more sensitive to variations in K^+ ion concentration.[5]

Sweadner provided the first concrete evidence for the existence of multiple forms of the Na^+K^+ATPase by isolating two forms of the enzyme from rat brain.[6] These two distinct proteins could be resolved by gel electrophoresis in sodium dodecyl sulfate (SDS). The two proteins also displayed differences in their sensitivity to trypsin digestion, in reactivity of sulfhydryl groups and in affinity for the cardiac glycoside strophanthidin, an aglycone analog of ouabain. Sweadner found that the isozyme of Mr 100,000, which she designated α+, localized only to the plasma membrane of myelinated axons. Conversely, the smaller isoform, designated α, was seen in all other tissue types examined as well as in non neuronal (glial) central nervous system and superior cervical ganglion cells.[6]

Subsequent research has provided more detailed information concerning the functional properties of these two α subunits. The α and α+ isoforms have been shown to differ in their interactions with phospholipids,[7] regulation by thyroid hormone[8] and insulin,[9] and inhibition by pyrithiamin.[10] The two isozymes of the α subunit have also been shown to contain distinct antigenic determinants.[11]

Recent studies have shown that the α subunit isoforms are more prevalent than originally reported. Matsuda and his colleagues found two forms of Na^+K^+ATPase α subunit in canine cardiac preparations that

could be separated by SDS electrophoresis and differed in their
inhibition by pyrithiamin.[10]   Recent work by Maixant et al., also
provided evidence of a second form of the α subunit in dog heart using
immunological techniques.   The two α subunit isoforms were both shown to
be active in dog heart.   The isoforms differed in their affinity for
ouabain and digitoxigenin by a factor of 150.[12]   Similar findings of
two molecular forms of Na^+K^+ATPase and two classes of ouabain receptors
have also recently been reported in ferret heart.[13]   Interestingly,
Matsuda failed to detect a second isoform in cardiac samples from a
number of other animals including guinea pig, rat, mouse, rabbit and
cow.[10]   McDonough and Schmitt were also unable to find a second form of
the enzyme in guinea pig heart.[14]   These results were especially
intriguing in the face of mounting evidence for the existence of two
populations of ouabain receptors in preparations from cardiac muscle
from several species including rat, dog, and guinea pig.[15,16,17,18]

Isoforms of the α subunit of the Na^+K^+ATPase have also been found
in tissues other than brain and heart.   Lytton described an insulin
sensitive isoform in rat adipocytes and skeletal muscle similar in size
and ouabain sensitivity to the α+ form described by Sweadner[9].   The
existence of α and α+ isoforms has also been reported in distinct
populations of mammalian ciliary cells,[19] in retina, and in optic
nerve.[20]

In addition to differences in functional properties and tissue
localization, experimental data suggests that the Na^+K^+ATPase varies in
location, prevalence, and function during development.   Details of the

variation remain controversial. Abdel-Latiff and Abood reported a more than six fold increase in Na^+K^+ATPase activity in nerve ending fractions from pre-natal to ten day old rats.[21] Conversely, Samson et al. found an absence of enzyme activity in the brains of newborn rats and then a rapid increase in activity between ten days and three weeks.[22] Cote reported detecting Na^+K^+ATPase activity in rat fetus as early as sixteen days of gestation.[23] Schmitt and McDonough found a ten-fold increase between the eighteenth day of gestation and twenty day old rat neonates and then a 1.8-fold increase between those neonates and adult rats.[24]

It has also been observed that several characteristics of the Na^+K^+ATPase differ between fetal and adult rat brains including specific activity [25,26] and temperature dependence.[27] Wilson described a number of other differences in Na^+K^+ATPase activity between these two age groups including disparate apparent K_m values of activation by K+ and ATP hydrolysis, activation by phosphatidyl choline, and relative inhibition by a fixed concentration of ouabain.[28] Recently, Schmitt and McDonough have used immunological techniques to detect a marked increase in the abundance of the α and α+ subunits in rat brain during pre- and neo-natal development which appears to be regulated by thyroid hormone.[24]

Developmental differences in α subunit expression have also been noted in other tissues. Newborn rat myocytes contain high and low affinity digitalis receptors that are not present in preparations from adult rat.[29,30] Changes in relative amounts of two molecular forms of α

subunit protein during development have also been described in brine shrimp.[31]

Recently, cDNAs have been isolated for the two Na^+K^+ATPase α subunit isoforms and their primary sequence has been deduced.[32,33] Surprisingly, a cDNA clone corresponding to a third rat Na^+K^+ATPase subunit, designated variously as αlll and α_2, has now also been isolated by several groups using cDNA cloning techniques[32,33]. The protein encoded by this gene has not yet been detected but the corresponding mRNA has been identified in rat brain, stomach and lung[34].

In contrast to the α subunit, only one form of the β subunit of the Na^+K^+ATPase has been isolated to date. The cDNA sequence has now also been established for the rat, torpedo, human, and dog β subunit genes. Their deduced amino acid sequences show a high degree of homology.[35,36,37] In rat the β subunit gene encodes four distinct mRNA species which are expressed in a tissue specific fashion. None of these species ,however, were detected by Northern blot analysis in preparations from rat liver.[38] Hubert et al. were also unable to detect the known form of this protein in rat hepatocytes using immunological techniques.[39] The absence of the Na^+K^+ATPase β subunit and expression of its corresponding mRNA in a tissue known to possess Na^+K^+ATPAse activity raises the possibility that other isoforms of the β subunit exist.

Recent work has shown that the three isoforms of the α subunit Na^+K^+ATPase are encoded by multiple genes which are located on separate chromosomes in the mouse.[40] The α or αl subunit gene (*Atpa-1*) is

located on chromosome three, the $\alpha2$ or αlll gene (*Atpa-2*) has been mapped to chromosome seven, and the gene for the $\alpha3$ or $\alpha+$ isoform (*Atpa-3*) is present on chromosome one. The β subunit gene (*Atpb*) is also located on chromosome one, but it is not tightly linked to the α subunit gene on that chromosome.[33] This information raises a number of questions regarding the control mechanisms used to generate the diversity seen in the enzyme's functional properties and activity level in various tissues and developmental periods.

Experimental evidence suggests that synthesis of the Na^+K^+ATPase α and β subunits is coordinately regulated. Using an immunological assay, Tamkum and Fambrough detected concurrent biosynthesis of α and β subunits in chick sensory neurons.[41] Similar findings have been reported in toad bladder tissue after stimulation with aldosterone.[42] Other studies have shown that in a variety of target tissues exposure to thyroid hormone results in an increase in Na^+K^+ATPase activity.[43] A study of kidney tissue from rats infused with triiodothyronine, however, showed an increase in synthesis of the α subunit protein with no corresponding increase in the protein identified as the β subunit.[44]

It has also been reported that expression of the mRNAs encoding the α and β subunits is coordinately controlled under certain conditions. After stimulation with aldosterone of a cell line derived from Xenopus kidney the induction of Na^+K^+ATPase activity was shown to be mediated by a two- to four-fold increased accumulation of both α and β subunit mRNAs.[45] Girardet et al. showed that in toad bladder cells either oxytocin or aldosterone administered alone increased the relative rate

of synthesis of the α and β subunits of the Na^+K^+ATPase. When both hormones were applied simultaneously a markedly increased effect was seen at the physiologic level. In contrast, no such synergistic effect was evident in the rate of mRNA synthesis.[46]

Other lines of investigation have produced similar discrepancies. Bowen and McDonough found that in cultured canine kidney cells grown in a low concentration of K^+, an increase in Na^+K^+ATPase activity could be attributed to a rapid and coordinate increase in α and β subunit mRNA which was followed by an increase in α and β subunit proteins as measured by immunoprecipitation.[47] In contrast, data obtained from experiments done on cultured chicken skeletal muscle by Wolitzky and Fambrough indicated that up regulation of Na^+K^+ATPase occurred by a combination of early stimulation of α and β subunit biosynthesis followed by a decrease in the enzyme's turnover rate.[48]

In a cell line derived from HeLa cells by mutagenesis and step-wise selection in ouabain, the development of a ouabain resistant phenotype has been demonstrated to be accompanied by a marked increase in expression of both the α and β subunits relative to the parent cell line.[49] Other studies, however, have attributed the ouabain induced increase in surface density of the Na^+K^+ATPase enzyme to a change in the rate constant for its turnover.[50]

In order to further investigate the tissue and developmental stage variation of the three α subunit isoforms and the β subunit of the Na^+K^+ATPase enzyme and to study the regulation of its biosynthesis I have examined the expression of the corresponding mRNAs in rat brain,

heart, liver and kidney at three representative stages of development. I have also studied the expression of the α and β subunit mRNAs in cell lines derived from both neuronal and glial elements of the rat central nervous system. Finally, I compared the levels of mRNA expression in the rat PC12 cell line grown with and without the presence of a nerve growth factor. PC12 cells are of pheochromocytoma origin and differentiate into cells resembling sympathetic neurons when induced by NGF.[51]

Substantial differences in expression of the mRNAs encoding the Na+K+ ATPase A and B isoforms were seen both in the various tissues examined and in the same tissues at different developmental stages. Moreover, these patterns were distinct and separate for the three α and the β subunit genes. Each of the three α isoforms was also expressed in all of the rat central nervous system (CNS) and pheochromocytoma cell lines. The β subunit mRNA was expressed only in the pheochromocytoma cells. These distinct patterns of expression of the mRNAs corresponding to the Na^+K^+ATPase subunits raises the possibility that the different members of this multi-gene family may have specialized functions and suggests that their regulation may be in part controlled by factors other than the level of RNA expression.

2. MATERIALS AND METHODS

Cell lines and Culture Conditions

Rat central nervous system (CNS) cell lines B35, B50, B60, and B103 (neuronal) and B15 and B82 (glial) were generously provided by Dr. David Schubert of the Salk Institute. The cells were maintained in Dulbecco's modified Eagle's medium supplemented with 15% fetal calf serum.

PC12 cells were kindly donated by the lab of Dr. Ed Hawrot of Yale Medical School. These cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 5% horse serum. A group of these cells was also grown for 9 days in 20 ng/ml nerve growth factor (Calbiochem).

Preparation of Total Cellular RNA

RNA was isolated from the rat CNS and PC12 cell lines and from fresh brain, heart, liver, and kidney of fetal (18 day), newborn (two week), and adult rats using the guanidinium isothiocyanate method described by Chirgwin et al..[52] In brief, freshly removed tissue was placed in 25 mls of guanidinium isothiocyanate stock, (4 M guanidinium isothiocyanate, 0.5% N lauroyl sarcosine, 25 mM sodium citrate, 0.1 M β-mercaptoethanol, and 0.1% Sigma 30% antifoam A), and immediately homogenized for one minute. The homogenate was then centrifuged for ten minutes at 8000 rpm. The supernatant was mixed with 0.6 ml of 1.0 M acetic acid and 18 mls of absolute ethanol and allowed to precipitate overnight at -20° C. This solution was then centrifuged at 6000 rpm for

ten minutes at -20° C. The pellet was resuspended in 15 mls of
guanidine hydrochloride stock solution, (7.5 M guanidine hydrochloride,
0.025 vol. 1.0 M sodium citrate, and 5 mM dithiothreitol). 0.4 ml 1.0 M
acetic acid and 8 mls ethanol was added to reprecipitate and the mixture
was kept at -20° C and centrifuged as above. A final reprecipitation
was performed with a further halving of all volumes and the resulting
solution was centrifuged at 6000 rpm for 5 minutes. The pellets were
then rinsed in ethanol and recentrifuged at 6000 rpm. Finally the RNA
was dissolved in sterile water and stored at -70° C.

Total cellular RNA from cultured cells was prepared using the same
procedure except that the guanidinium isothiocyanate solution was
applied to monolayers of each of the cell types and allowed to stand for
five minutes before the mixture was homogenized.

RNA Blot Hybridization

Total cellular RNA (20 μg) was denatured by heating at 65° C for
ten minutes in 50% (vol/vol) formamide and was fractionated by
electrophoresis through a 1% agarose formaldehyde gel.[53] The RNA was
then transferred to reusable hybridization membranes (Zetabind, AMF
Cuno).

The RNA blots were prehybridized for 24 hours at 42° C. The
prehybridization buffer consisted of 50% (vol/vol) formamide, 5x sodium
chloride, 50 mM sodium phosphate, sonicated, denatured salmon sperm DNA
at 250 μg/ml, and 0.02% each bovine serum albumin, ficoll, and
polyvinylpyrrolidone.[54,55] The hybridization buffer contained four

parts prehybridization buffer and one part 50% (wt/vol) dextran sulfate as well as 100 μg/ml each of single stranded poly(A), poly(I) and poly(C) ribonucleotides at 100μg/ml each (Boehringer-Mannheim). Radiolabeled DNA probes were synthesized using the Klenow fragment of DNA polymerase 1 with random hexanucleotides (Boehringer-Mannheim) and $[\alpha-^{32}P]$ dCTP.[56]

The RNA blots were hybridized with 10^7 cpm (<50 ng) of the various hybridization probes for 24 hours and were then washed to a final stringency of 15 mM sodium chloride, 1.5 mM sodium citrate, and 0.1% SDS at 55° C and exposed to Kodak XRP film at -80° C with an intensifying screen. For reprobing, blots were washed twice for 15 minutes in 15 mM sodium chloride, 1.5 mM sodium citrate, 1.3 mM KH_2PO_4, 100μM EDTA and 0.1% SDS at 95° C.

cDNA Probes

A map of the rat Na^+K^+ATPase α and β subunit cDNA clones is shown in Figure 1. Each of the RNA blots was probed with the following coding regions from the three Na^+K^+ATPase α subunit isoforms:

1. A 3.2 kilobase pair (kbp) rat cDNA containing the entire coding region from the α (α1) subunit gene (*Atpa-1*).

2. A 1.8 kbp Eco RI fragment of the rat cDNA encoding the amino terminal portion of the αlll (α3) subunit gene (*Atpa-2*).

3. A 2.7 kbp rat cDNA encoding a segment of the α+ (α3) subunit gene (*Atpa-3*).

In addition, each blot was also probed with a 1.2 kbp cDNA containing the entire coding region of the rat $Na^+K^+ATPase$ β subunit gene ($Atpb$).

As a control each blot also was hybridized with a human gamma actin cDNA probe (pHFγ A) kindly supplied by Dr. Larry Kedes of Stanford University School of Medicine.
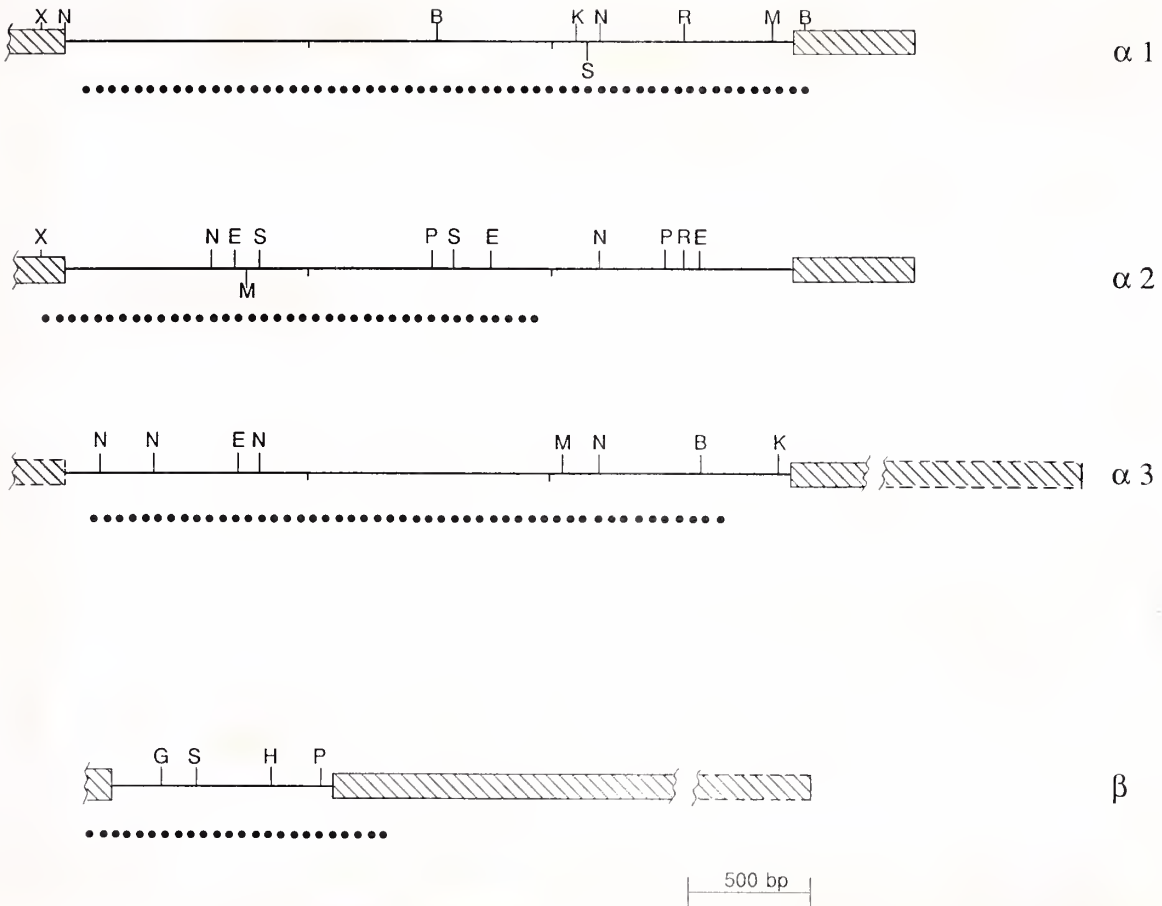
Figure 1. Map of rat Na$^+$K$^+$ATPase α and β subunit cDNA clones. Solid lines represent the open reading frame and hatched boxes represent the untranslated regions. Dotted lines represent the fragement of a clone that was used as a probe. Restriction sites: B, *Bam*HI; E, *Bst*EII: G, *Bgl*I; H, *Hind*III; K, *Kpn* I; M, *Mst*II; N *Nco* I; P, *Pst* I; R, *Eco*RI; S, *Stu I; X, Rsr* II. bp, Base pairs.

3. <u>RESULTS</u>

<u>Tissue and Developmental Stage Specific Expression of Na$^+$K$^+$ATPase α Subunit mRNAs</u>

Expression of the mRNAs encoding the three known isoforms of the Na$^+$K$^+$ATPase was examined by sequentially hybridizing cDNA probes specific for each of the isoforms to a Northern blot containing total cellular RNA from rat brain, heart, kidney and liver at three different stages of development. Previous experiments using these probes have shown that virtually no cross hybridization occurs in Southern genomic blots hybridized under the same conditions.[33] The results of these Northern blots are shown in Figure 2.

The α1 subunit cDNA probe hybridized to an approximately 4.5 kb RNA species as shown in the α1 panel. In adult rat, transcripts of the *Atpa-1* gene were most prevalent in kidney. Fewer transcripts were apparent in adult heart and brain. Even fewer transcripts were expressed in the RNA from adult liver tissue. *Atpa-1* mRNA was also abundant in newborn (two week) brain, heart, and kidney as well as fetal (18 day) heart and kidney. It was less abundant in fetal brain and liver and newborn liver.

A different pattern of expression was seen for the *Atpa-2* gene which encodes the α2 or αIII subunit although it also hybridized to an mRNA of approximately 4.5 kb in length. As shown in the α2 panel of Figure 2, this mRNA was most prevalent in newborn and adult brain and in newborn liver. Moderate levels of *Atpa-2* mRNA were detected in adult

kidney and liver and newborn kidney. In heart tissue at all the developmental stages examined and in fetal kidney a very low level of expression was seen. No transcripts of the *Atpa-2* gene were visible in fetal brain or liver.

The results of hybridization of the Northern blot with the cDNA which encodes the $\alpha 3$ or $\alpha+$ subunit is also shown in Figure 2 (panel $\alpha 3$). The *Atpa-3* gene hybridizes to three distinct mRNA species of approximately 6.0, 4.5, and 4.0 kb in length. These multiple mRNA species are consistent with the published sequence data for the $\alpha+$ gene which contains three possible sites for polyadenylation.[32] Transcripts of the 6.0 kb mRNA were most abundant in newborn and adult brain. They were less abundant in fetal heart and kidney, in newborn kidney and liver, and in adult heart and liver. The 4.5 kb mRNA was expressed predominately in two week brain. Lower levels of expression of this gene were observed in fetal heart, newborn heart, kidney and liver, and in adult rat kidney and liver. The 4.0 kb mRNA species had the most restricted pattern of expression. It was detected only in fetal kidney and in adult brain and heart.

The same Northern blot showed bands of approximately equal intensity when probed with actin cDNA. The differences in intensity of hybridization seen with the various α subunit probes are therefore probably a reflection of variation in level of mRNA expression. These results demonstrate that the three known $Na^+K^+ATPase$ α subunit isoform mRNAs are expressed in distinct tissue and developmental stage specific distribution patterns.

Tissue and Developmental Stage Specific Expression of the Na$^+$K$^+$ATPase β
Subunit mRNA

The results of hybridizing the Northern blot of rat tissues with a
probe of the Na$^+$K$^+$ATPase β subunit cDNA are shown in the β panel of
Figure 2. The *Atpb* gene encodes four distinct mRNA species which range
from approximately 3.0 to 2.0 kb in length. Transcripts of the β mRNA
were most abundant in adult brain and newborn liver. A lower level of
expression was seen in fetal and adult kidney and the lowest level was
observed in newborn brain and kidney and in heart tissue at all three
developmental stages. No hybridization was detected to the RNA from
fetal brain and liver or adult liver.

The same blot probed with actin cDNA revealed a single mRNA species
that hybridized to all lanes with approximately equal intensity (Figure
2, actin panel). These results demonstrate that the Na$^+$K$^+$ATPase β
subunit gene *Atpb* is also expressed in a tissue and developmental
specific fashion and that its pattern of expression differs from that
seen for any of the genes encoding isoforms of the α subunit of that
enzyme.

Expression of the mRNAs Encoding the Na^+K^+ATPase α and β Subunits in Cell Lines Derived from Components of the Rat Nervous System

The various isoforms of the Na^+K^+ATPase enzyme may be localized in specific cell types or regions within the central nervous system.[6,19,20] It has also been shown that superior cervical ganglion cells of the peripheral nervous system, in contrast to neuronal cells of the CNS, do not contain detectable levels of the α+ form of the α subunit.[6]

Several lines of experimentation have shown that cultured cell lines derived from neural and glial components of the CNS may serve as reasonable models for the ion transport properties of their tissue counterparts.[57,58] It has also been established that the PC12 cell line, which is derived from rat adrenal pheochromocytoma cells of neural crest origin, develops the morphology and many of the characteristics of sympathetic neurons when it is grown in the presence of nerve growth factor.[51] To ascertain whether variation exists in pattern of mRNA expression in specific cell types within the nervous system, a Northern blot containing total cellular RNA from four cell lines of neuronal derivation (B35, B50, B60 and B103) and two cell lines of glial origin (B15 and B82)[59] as well as RNA from PC12 cells cultured with and without nerve growth factor was hybridized with cDNA probes of Na^+K^+ATPase α and β subunits. The results are shown in Figure 3.

Transcripts of the *Atpa-1* and *Atpa-2* genes were expressed in all of the cell lines examined (panels α1 and α2). The *Atpa-3* gene was also expressed in each of the cell lines as shown in panel α3. The 4.5 kb mRNA species of this gene was detected in the B15, B35, B60, B82, B103

and PC12 cells. In contrast, the 6.0 and 4.5 kb mRNA species of this α subunit isoform were apparent only in RNA from the B50 cells.

A markedly different set of data was obtained when the same Northern blot was hybridized with a cDNA probe coding for the Na^+K^+ATPase β subunit. Transcripts of the *Atpb* gene were seen only in PC12 cells (Figure 3, panel β).

As shown in the actin panel of Figure 3, an actin cDNA probe applied to this Northern blot produced hybridization of approximately equal intensity in the lanes corresponding to each of the cell lines. The inability to detect β subunit mRNA transcripts in the majority of these cell lines is thus probably caused by either a low level or complete lack of expression of the β subunit gene in those cells instead of RNA degradation or a flaw in the hybridization technique used.

Of note is that the PC12 cell line showed striking differences in expression of the α and β subunit mRNAs when grown in the presence of NGF. The $\alpha1$ cDNA probe produced hybredization of approximately equal intensity for the PC12 cells grown with or without NGF. The intensity of the $\alpha2$ cDNA probe, however, markedly decreased in the lane corresponding to cells grown with NGF. A down-regulation of β subunit mRNA was also seen in the NGF-induced PC12 cells. In contrast, a much higher intensity of hybridization was apparent with the mRNA from the NGF-induced PC12 cells when the $\alpha3$ probe was used.

The patterns of expression of the Na^+K^+ATPase α and β subunits seen in these cell lines of nervous system origin indicate that expression of the three α subunits is fairly widespread within the components of that

tissue.   In contrast, expression of the β subunit gene is much more circumscribed.   Moreover, the differences in expression of the α and β subunit mRNAs between PC12 cells grown in standard media and those induced by NGF imply that expression of these mRNAs changes as a cell differentiates.
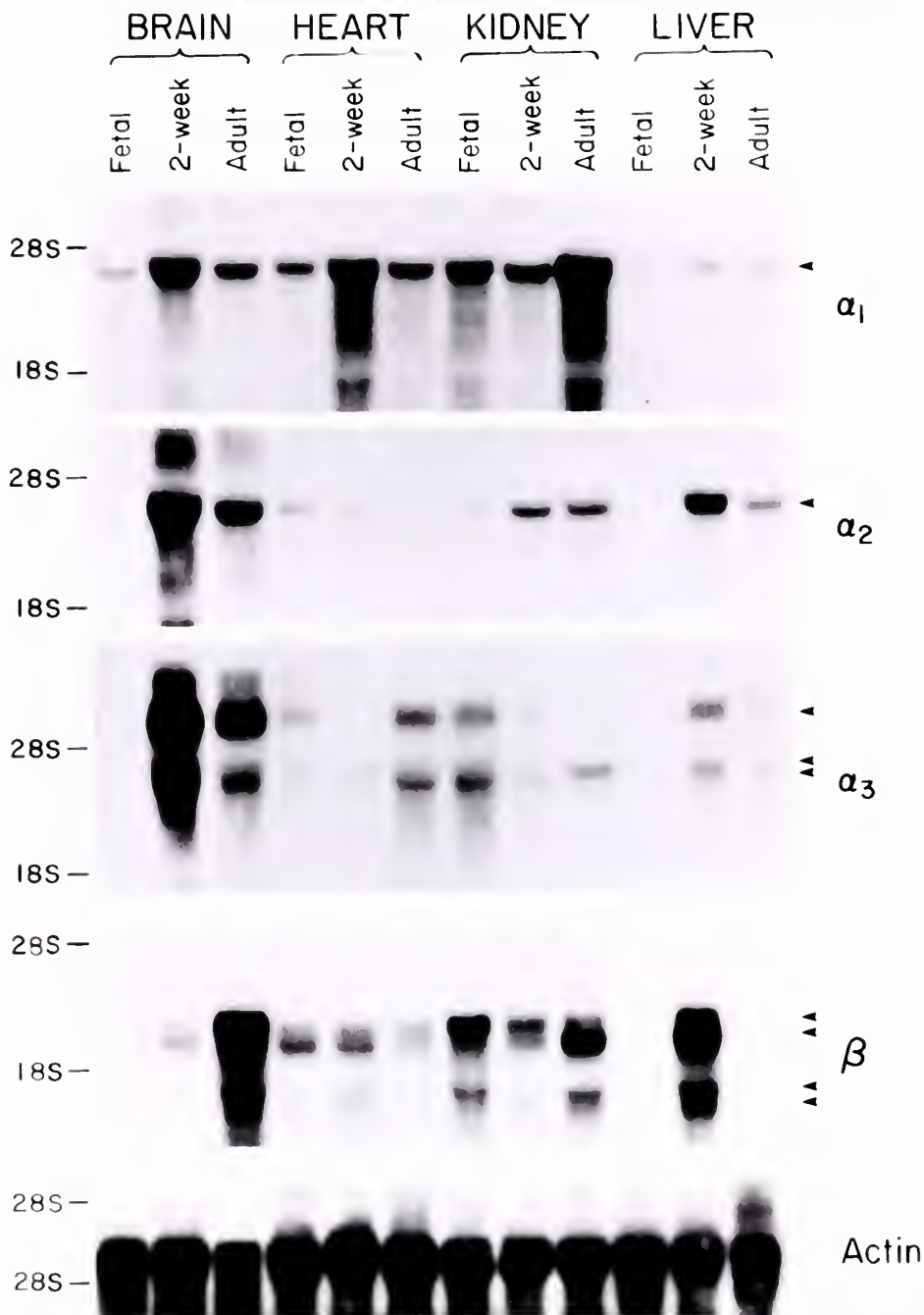
Figure 2. Expression of the Na^+K^+ATPase α and β subunit genes in rat tissues. Total cellular RNA (20 μg) was prepared from fetal (18 day), two week old, and adult rat tissues and sequentially hybridized with the cDNA probes indicated on the right. The positions of the 28S and 18S markers are indicated on the left. The arrowheads on the right denote the positions of the α and β subunit mRNA sequences.
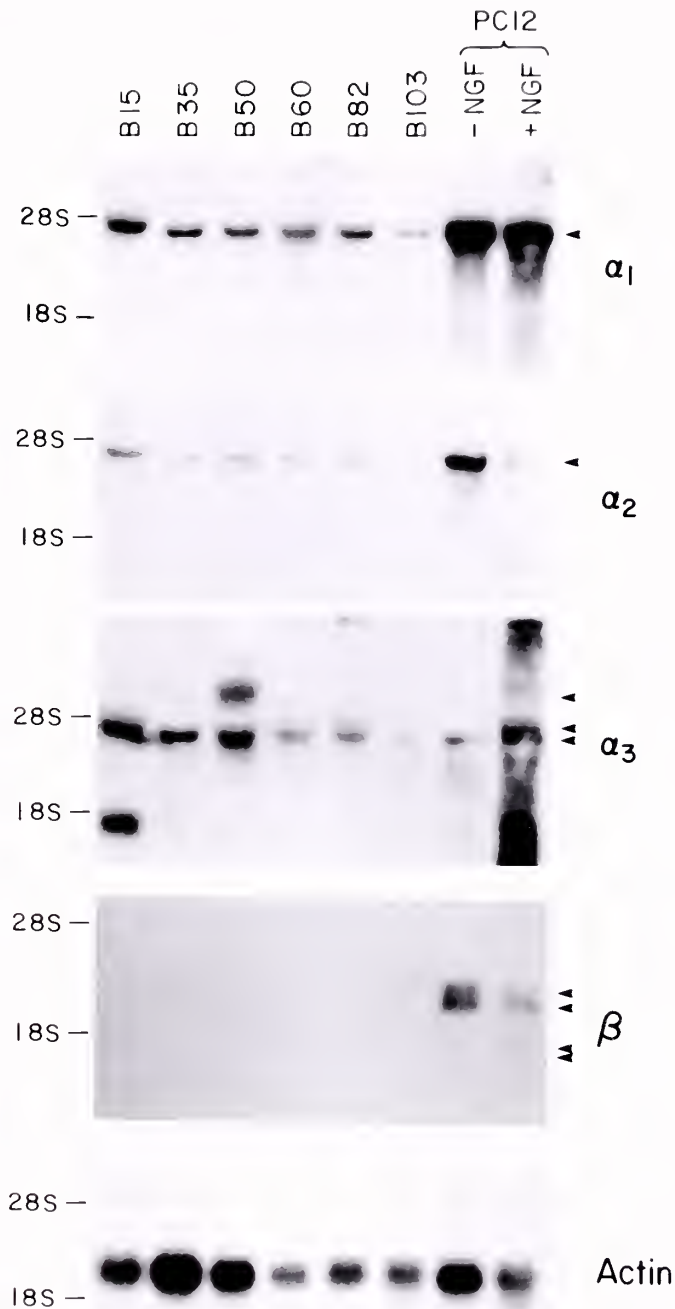
Figure 3. Expression of the Na$^+$K$^+$ATPase in rat cell lines. Total cellular RNA (20 μg) prepared from the rat CNS cell lines described in the text and from PC12 cells growth in the absence (-NGF) or presence (+NGF) of nerve growth factor were sequentially hybridized with the cDNA probes indicated on the right. The positions of the 28S and 18S markers are indicated on the left. The arrowheads on the right denote the positions of the α and β subunit mRNA sequences.

4. DISCUSSION

For a number of years investigators have produced data showing that variation exists in the activity level and character of the Na^+K^+ATPase enzyme both in various tissues of the same species and in the same tissue at different developmental stages. There are, however, a number of discrepancies in this body of data. Studies of this ubiquitous enzyme have indicated that the biosynthesis of its α and β subunits is coordinately regulated and that changes in the rate of subunit biosynthesis is the major control mechanism of enzyme activity. Data also exist that contradict this hypothesis. The discovery of various isoforms of the Na^+K^+ATPase enzyme and the molecular cloning of the cDNAs encoding them have provided the means for a more in depth investigation of both the molecular correlates of the tissue and developmental stage specific variation observed and of the control mechanisms regulating this diversity.

The discovery that the α subunits of the Na^+K^+ATPase exist as a multigene family with at least three members provides a possible explanation for many of these discrepancies in data concerning the functional properties and activity level of the enzyme. Many of the assays traditionally used to ascertain the presence of the Na^+K^+ATPase enzyme employ characteristics such as cardiac glycoside binding or presence of certain antigenic determinants that have been shown to differ in at least two of the known isoforms of the α subunit. It is therefore possible that previous studies may have relied on assays that

were preferentially sensitive to a particular isoform and for that reason produced spurious or contradictory results. My experimental data indicates that the β and various α subunit mRNAs each display distinct pattern of expression in the tissue types and developmental periods examined. The isolation of new members of this multigene family and the proteins encoded by them should enable many of the existing ambiguities concerning distribution of the enzyme to be resolved.

The discovery that the Na^+K^+ATPase enzyme exists in a number of forms that differ radically in their distribution raises several other issues that may prove much more difficult to address. Perhaps the most intriguing of these questions is that of the functional correlates to the observed diversity. It would seem a reasonable hypothesis that the various isoforms have evolved to satisfy a need for divergent functions of the Na^+K^+ATPase enzyme.

Several lines of evidence indicate that the isoforms of the enzyme fulfill a variety of roles. This diversity is perhaps best illustrated in the nervous system. It has been demonstrated that the appearance of the Na^+K^+ATPase correlates well with the onset of electrical activity in rat brain.[60] In neurons, the enzyme is found in especially high concentrations and is responsible for maintaining the high ionic gradients necessary for both the generation of nerve impulses and neurotransmitter uptake.[61] Glial cells have been shown to contain a form of the Na^+K^+ATPase enzyme that is more sensitive to variations in K^+ concentration than the neuronal form, a property well suited to the function of these cells in regulating the concentration of the K^+ ion in

the extracellular space surrounding the neurons.[62,5] These functional

differences correlate well with published data that the $\alpha+$ isoform of

the $Na^+K^+ATPase$ enzyme is found in myelinated axons whereas in non-

neuronal cells of the CNS the $\alpha1$ subunit is present. One potential

problem with this assignment of specific functions to these α subunit

isoforms in nervous system tissue is that neurons of the sympathetic

nervous system appear to contain only the $\alpha1$ isozyme.[6] It has also been

demonstrated that the $\alpha1$ and $\alpha+$ isoforms are present in distinct ciliary

epithelial and optic nerve cell populations where their differences in

function are less clear.[19,20] My results indicate that in addition to

the α and $\alpha+$ isoforms the $\alpha2$ (or $\alpha111$) subunit gene is also expressed

at fairly high levels in developing and adult rat brain. More

experiments are needed to determine whether the protein encoded by this

gene is also present in brain tissue and what, if any, specialized

functions it performs.

Curiously, in the cell lines derived from neuronal and glial

elements and in the nerve growth factor induced pheochromocytoma (PC12)

cells expression of each of the three α subunit isoforms was observed,

albeit at somewhat varying levels. One possible explanation is that the

cultured cell lines had in some way de-differentiated and were therefore

displaying properties characteristic of more pluripotent cell types.

This is unlikely to be the case for two reasons. First, not all of the

isoforms were detected in RNA from fetal rat brain tissue which is

ostensibly not yet fully differentiated. Second, a more circumscribed

pattern is seen with β subunit expression in the same cultured cell

lines. Another possibility is that all of the Na^+K^+ATPase α subunit
genes are transcribed in nervous system cells and then only specific
mRNAs are translated. Another possible scenario is that multiple
isoforms can exist in a given cell type, perhaps with specific
subcellular localization. Data showing approximately equal synthesis of
the α1 and α+ subunit isoforms in optic nerve but axonal transport of
predominantly the α+ form is consistent with such a system.[20] This
theory, however, is challenged by my data showing that PC12 cells up and
down regulate subunit mRNA transcription when induced by NGF. More
specific assays could provide useful information concerning the exact
cellular and subcellular localization of the various Na^+K^+ATPase
isozymes within the nervous system, as well as new insights into the
relationship between localization and function in that organ system.

Specialized functions of the Na^+K^+ATPase enzyme isoforms have been
identified in other tissues as well as in the nervous system. In dog
heart, it has been demonstrated that the α+ isoform is responsible for
the positive inotropic effect of the cardiac glycosides whereas the α1
isoform has been implicated in the toxicity associated with those
agents.[12] Studies in ferret heart indicate, however, that the α1 and α+
isoforms are associated with high and low affinity ouabain binding sites
and that ouabain can interact with either of these receptors to produce
its positive inotropic effect.[13] In rat adipocytes it has been shown
that both the α and α+ isoform are present but only the α+ form is
sensitive to stimulation by insulin.[9] My data showing significant
differences in the expression of the three α subunit isoform and β

subunit mRNAs in rat heart, kidney, and liver tissue support the idea that specialized function is related to preferential expression of certain isoform mRNAs.

Experiments on the physiological characteristics of the Na^+K^+ATPase during various stages of development have shown differences in some tissues. Charlemagne et al. showed that in newborn rat heart ouabain is bound and released at a rate four to five times slower than in normal adult rat heart. Interestingly, a similar rate was observed in myocardium that had hypertrophied in response to mechanical stress.[63] Paradoxically, in my experiments heart tissue displayed the least variation in expression of Na^+K^+ATPase subunit mRNAs of any tissue examined. In brain tissue a similar discrepancy exists between level of expression and observed physiological differences in functional properties and activity level. Schmitt and McDonough demonstrated a ten fold increase in Na^+K^+ATPase activity between 18 day fetal rats and those 20 days old.[28] My data showed an approximately five to ten fold increase in α subunit mRNA between fetal and two week old rats. Expression of the β subunit, however, increased even more in the same time period. These results warrant further investigation of the activity levels of the Na^+K^+ATPase enzyme during various stages of maturation in different tissue types.

My data show marked variation in mRNA expression of the Na^+K^+ATPase α and β subunits in various tissue types at different developmental stages. This diversity, as well as the known variation in enzyme character and activity level, cannot be explained solely by

levels of mRNA expression; it raises the question of what control

mechanisms generate this tremendous diversity. Experiments have shown

that the α and β subunits are coordinately synthesized and that control

of enzyme activity is effected largely by changes in the synthesis rate

of the mRNAs encoding the subunit proteins.[42,46,48] The discovery that

the genes for the α subunit isoforms and the β subunit lie on separate

chromosomes makes it unlikely that a common cis acting control mechanism

is responsible. My experimental data strongly suggest that the

diversity observed on the physiological level cannot be explained solely

on the basis of level of gene expression and therefore support the idea

that other factors play a contributory role in the control of

biosynthesis of the Na^+K^+ATPase enzyme. Although changes in turnover

rate constant has been implicated by several lines of

experimentation,[48,50] my data suggest another possibility. I have shown

that the mRNA of the known β subunit is not expressed in liver or in any

of a number of cell lines derived from glial or neuronal components of

the central nervous system or from the sympathetic nervous system.

These data, as well as previous work of several investigators who were

unable to detect either the protein or the mRNA corresponding to the β

subunit in liver tissue,[35,39] support the hypothesis that either as yet

undetected isoforms of the β subunit exist which differ considerably

from the known form or that the β subunit is not always a necessary

component for enzymatic activity in some tissues. This speculation is

at variance with the established belief that the active form of the

enzyme exists as some multiple of an $\alpha\beta$ dimer, but raises another

possibility for a diversity-generating control mechanism. It is possible that enzyme activity level and function may be determined by different combinations of α and β subunit isoforms or α subunit isoforms functioning alone. This theory is supported by recent work of Numa and his colleagues showing that Xenopus oocytes injected with the Na^+K^+ATPase α and β subunit mRNAs produced more α subunit polypeptides than oocyctes injected with the same concentration of α subunit mRNA alone.[64]

Identification of the cDNAs encoding the Na^+K^+ATPase α and β subunit isoforms and the isolation of their associated proteins are required to provide more satisfying answers to the related issues of function and control raised by the variability in distribution, functional properties, and mRNA expression of this enzyme. The development of systems to transfer and express the genes encoding Na^+K^+ATPase subunit isoforms in bacteria or other lower organisms, as well as exploitation of characteristics such as varying sensitivity to cardiac glycosides, should provide researchers with data needed to clarify the function and biosynthesis of this complex enzyme.

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